Mutagenic Evaluation of Compound FD \ \4 **Ferrous Sulfate** 10/29/74

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LBI PROJECT #02468

MUTAGENIC EVALUATION OF COMPOUND FDA 71-64 FERROUS SULFATE

SUBMITTED TO

FOOD & DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE
ROCKVILLE, MARYLAND

SUBMITTED BY

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Litton BIONETICS

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EVALUATION SUMMARY

Compound FDA 71-64, ferrous sulfate, was found to be activated to a genetically active agent in suspension tests with mouse, rat and monkey tissues. The results indicate that the active agent is a frameshift mutagen which strongly reverts strain TA-1537. Rat liver tissue appeared to be the most effective activating system. The yeast indicator organisms also appeared to respond to ferrous sulfate although those results are questionable. No genetic activity was observed in plate test assays.

DATE: October 1, 1974

SPONSOR: Food and Drug Administration, Contract Number 223-74-2104

SUBJECT: Mutagenic Evaluation of Compound FDA 71-64

I. OBJECTIVE

The objective of this study was to assess the genetic activity of the test material in microbial assays with and without the addition of mammalian metabolic enzyme preparations.

II. MATERIALS

A. Test Material

Ferrous sulfate

Mallinckrodt WZ PV

B. Tissue Homogenates and Supernatants

The tissue homogenates and 9,000 x g supernatants were prepared from liver, lung and testes of the following mammalian species: Mouse - ICR random bred adult males; rat - Sprague-Dawley adult males; and primate - Macaca mulatta adult males.

C. Indicator Organisms

The indicator organisms used for all tests are described below:

- Saccharomyces cerevisiae, strain D4: $\frac{\alpha}{a}$, $\frac{\text{ade 2-2}}{\text{ade 2-1}}$, $\frac{\text{try 5-12}}{\text{try 5-27}}$

- Salmonella typhimurium, strains:

  TA-1535; hisG, uvrB, rfa (missense mutation)
  TA-1537; hisC, uvrB, rfa (- frameshift mutation)
  TA-1538; hisD, uvrB, rfa (+ frameshift mutation)

D. Reaction Mixture

The following reaction mixture was employed in the activation tests:

	Component	Final Concentration/ml
1.	TPN (sodium salt)	6 µM
2.	Isocitric acid	49 µM
3.	Tris buffer, pH 7.4	28 µM
4.	$MgCl_2$	1.7 µM
5.	Isocitric dehydrogenase	6.3 Units
6.	Tissue homogenate or cell fraction	72 mg

Components 1-4 were combined and frozen as a "core" reaction mixture to which the other components were added just prior to use.

E. Positive Control Compounds

Table 1 lists chemicals for positive controls in the direct and activation assays.

TABLE 1

POSITIVE CONTROLS USED IN DIRECT AND ACTIVATION ASSAYS

ASSAY	CHEMICAL[a]	SOLVENT	PROBABLE MUTAGENIC SPECIFICITY[b]
Non-activation	Ethylmethane sulfonate	Water or saline	BPS
	2-Nitrosofluorene	Dimethylsulfoxide[c]	FS
	Quinacrine or Quinacrine mustard	Water or saline	FS
Activation	Dimethylnitrosamine	Water or saline	BPS
	2-Acetylaminofluorene	Dimethylsulfoxide[c]	FS

[a] Concentrations given in the Results Section.

[b] BPS = base-pair substitution; FS = frameshift.

[c] Previously shown to be non-mutagenic, see Appendix.

III. METHODS

A. Toxicity

The solubility, toxicity and doses for all chemicals were determined prior to screening.

Each chemical was tested for survival against strains TA-1537 and D4 over a range of doses to determine the 50% survival dose. Bacteria were tested in phosphate buffer, pH 7.4, for one hour at 37°C on a shaker. Yeasts were tested in phosphate buffer, pH 7.4, for four hours at 30°C on a shaker. The 50% survival dose was determined from the survival curve and the 1/4 and 1/2 50% doses calculated.

If no toxicity was obtained for a chemical with a given strain, then a maximum dose of 5% (w/v) was used against the strain.

Unless otherwise specified, the doses calculated for the tests in buffer were applied to the activation tests. The solubility of the test chemical under treatment conditions is stated in the Results Section.

B. Plate Tests

Only three bacteria strains were tested in qualitative plate tests. In the non-activation procedure, approximately 10^9 cells of a log phase culture of the bacterial indicator strains were spread over the surface of a minimal plate, and a measured amount of the test chemical was placed in the center of the test plate. In activation tests, the test chemical was added to the cells, and an aliquot of the mixture was spread on the surface of the test plate. The reaction mixture (0.1 ml) plus tissue extract was then spotted on the surface of the plate. Positive and solvent controls were included. All plates were incubated at 37°C for four days and then scored. Each compound (Test, Positive Control and Solvent Control) was done in duplicate. The results were scored as + or -. Concentrations of the positive control compounds are listed in the Results Section.

C. Suspension Tests

1. Non-activation

Log-phase bacteria and stationary-phase yeast cultures of the indicator organisms were grown in complete broth, washed and resuspended in 0.9% saline to densities of 1×10^9 cells/ml and 5×10^7 cells/ml, respectively. This constituted the working stock for tests of a group of test chemicals and their respective controls. Tests were conducted in 30 ml plastic tissue culture flasks. Cells plus appropriate volume(s) of the test chemical were added to the flasks to give a final volume of 2 ml. Solvent replaced the test chemical in the negative controls. Treatment was at 30°C for four hours for yeast tests and at 37°C for one hour for bacterial tests. All flasks were shaken during treatment. Following treatment, the flasks were set in ice. Aliquots of cells were removed, diluted in sterile saline (4°C) and plated on the appropriate complete media. Undiluted samples from flasks containing the bacteria were plated on minimal selective medium. Samples from a 10^{-1} dilution of treated cells were plated on the selected media for enumeration of gene conversion with strain D4. Bacterial plates were scored after incubation for 48 hours at 37°C. The yeast plates were incubated at 30°C for 3-5 days before scoring.

2. Activation

Bacteria and yeast cells were grown and prepared as described in the non-activation tests except that the cell densities were increased approximately five-fold for working stock suspensions. Measured amounts of the test and

control chemicals plus 0.25 ml of the stock cell suspension were added to a 30 ml plastic tissue homogenate. All flasks (bacteria and yeast) were incubated at 37°C with shaking. The treatment times as well as the dilutions, plating procedures and scoring of the plates were the same as described for non-activation tests.

D. Preparation of Tissue Homogenates and 9,000 x g Cell Fractions

1. Mice

Male mice (sufficient to provide the necessary quantities of organs for testes, lung and liver homogenates) were killed by cranial blow, decapitated and bled. The three organs were immediately dissected from the animal using aseptic techniques and placed in ice-cold 0.25 M sucrose buffered with Tris at pH of 7.4. Upon collection of the desired quantity of organs, they were washed twice with fresh buffered sucrose and completely homogenized with a motor-driven homogenizing unit at 4°C. The whole organ homogenate obtained from this step was divided into two samples. One sample was frozen at -80°C and the other was centrifuged for 20 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from the centrifuged sample was retained and frozen at -80°C. These two frozen samples were used for the activation studies.

2. Rats

The same procedures as described for mice was used for this mammal.

3. Primates

The liver, lungs and testes were aseptically removed from freshly killed adult male rhesus (M. mulatta) monkeys. Each organ was cut into a number of pieces each sufficient for one week's studies. The tissues were labeled and frozen at -80°C until needed. Tissue homogenates and 9,000 x g supernatants were prepared as described for mice.

E. Data Recording and Reporting

Following the specified incubation periods all population plates were scored by an automatic colony counter and the results from each plate of a set was recorded, in ink, in bound data books. Information necessary for identification of the specific experiment as well as the presence of any contaminant micro-organisms was recorded with each set of plate counts. All minimal or other types of selective media plates were hand scored and the results recorded along with the respective population data. For bacteria strains the number of colonies recorded from either the population or selective plates represents that number in 1 ml of test suspension plated. The numbers recorded for the yeast strain D4 represent the number in 0.5 ml of test suspension plated.

Frequencies were mechanically calculated and double checked. All data presented in the Results Section of this report consists of the actual sum of all raw data plate counts and only the frequencies are calculated figures.

IV. SOLUBILITY PROPERTIES OF THE TEST COMPOUND

1. NAME OR DESCRIPTION OF TEST COMPOUND: FDA 71-64

   FERROUS SULFATE

2. TEST SOLVENT AND DESCRIPTION OF SOLUBILITY OF THE TEST CHEMICAL UNDERTREATMENT CONDITIONS: 0.067 M phosphate buffer, pH 7.4, was used as the solvent for this compound, and the compound was soluble under treatment conditions in all tests.

3. OTHER COMMENTS:

V. TOXICITY AND DOSAGE DETERMINATIONS

COMPOUND FDA 71-64

		D4	TA-1537
	Dose No.	% Concentration	% Concentration
Range of concentrations of the test compound used to determine the 50% survival level	1	1	1
	2	2	2
	3	3	3
	4	4	4
	5	5	5
	Dose No.	% Survival	% Survival
Survival Results	Control	100	100
	1	100	52
Test Date: 7-8-74	2	100	15
	3	100	3
	4	94	0
	5	83	0
	Dose	% Concentration	% Concentration
Concentrations of the test chemical required for mutagenicity tests	Plate Test	-	0.50%
	¼ 50% Survival	2.0%	0.25%
	½ 50% Survival	4.0%	0.50%
	Other 50% s	8.0%	-

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VI. NON-ACTIVATION PLATE TESTS

DATE: 8-30-74

Test	Compound	Concentration/plate	TA-1535 T-1	TA-1535 T-2	TA-1537 T-1	TA-1537 T-2	TA-1538 T-1	TA-1538 T-2
PC	EMS	0.05 ml undiluted chemical	+	+				
	QM	0.25 mg			+	+		
	NF	0.25 mg					+	+
SC	SALINE	-	-	-	-	-		
	DMSO	<10%					-	-

NOTE: PC = positive control
SC = solvent control
T-1 = trial 1
T-2 = trial 2
EMS = ethyl methanesulfonate
QM = quinacrine mustard
NF = nitrosofluorene
DMSO = dimethyl sulfoxide
(c) = contamination present

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Litton BIONETICS

NON-ACTIVATION PLATE TESTS

DATE: 8-30-74

Test	Compound	Concentration	TA-1535		TA-1537		TA-1538	
			T-1	T-2	T-1	T-2	T-1	T-2
TC	FDA 71-64	0.50%	-	-	-	-	-	-

NOTE: TC = test compound
T-1 = trial 1
T-2 = trial 2

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VII. ACTIVATION PLATE TESTS

SPECIES: MOUSE

DATE: 8-30-74

Test	Organ	Compound	Concentration/plate	TA-1535 T-1	TA-1535 T-2	TA-1537 T-1	TA-1537 T-2	TA-1538 T-1	TA-1538 T-2
PC	Li	DMNA	25 µmoles	+	+				
		AAF	1.25 mg			+	+	+	+
	Lu	DMNA	25 µmoles	+/-	+/-				
		AAF	1.25 mg			-	-	-	-
	T	DMNA	25 µmoles	-	-				
		AAF	1.25 mg			-	-	-	-
SC	-	DMNA	25 µmoles	-	-				
	-	AAF	1.25 mg			-	-	-	-
	-	Saline	-	-	-				
	-	DMSO	<10%			-	-	-	-

NOTE:
PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2
DMSO = dimethyl sulfoxide
(c) = contamination present

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Litton BIONETICS

ACTIVATION PLATE TESTS

SPECIES: MOUSE

DATE: 8-30-74

Test	Organ	Compound	Concentration	TA-1535		TA-1537		TA-1538	
				T-1	T-2	T-1	T-2	T-1	T-2
TC	Li	FDA 71-64	0.50%	-	-	-	-	-	-
	Lu	FDA 71-64	0.50%	-	-	-	-	-	-
	T	FDA 71-64	0.50%	-	-	-	-	-	-

NOTE: TC = test compound
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2

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ACTIVATION PLATE TESTS

SPECIES: RAT

DATE: 8-30-74

Test	Organ	Compound	Concentration/plate	TA-1535 T-1	TA-1535 T-2	TA-1537 T-1	TA-1537 T-2	TA-1538 T-1	TA-1538 T-2
PC	Li	DMNA	25 µmoles	+	+				
		AAF	1.25 mg			+	+	+	+
	Lu	DMNA	25 µmoles	-	-				
		AAF	1.25 mg			-	-	-	-
	T	DMNA	25 µmoles	-	-				
		AAF	1.25 mg			-	-	-	-
SC	-	DMNA	25 µmoles	-	-				
	-	AAF	1.25 mg			-	-	-	-
	-	Saline	-	-	-				
	-	DMSO	<10%			-	-	-	-

NOTE:
PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2
DMSO = dimethyl sulfoxide
(c) = contamination present

Project No. 02468

ACTIVATION PLATE TESTS

SPECIES: RAT

DATE: 8-30-74

Test	Organ	Compound	Concentration	TA-1535 T-1	TA-1535 T-2		TA-1537 T-1	TA-1537 T-2		TA-1538 T-1	TA-1538 T-2
TC	Li	FDA 71-64	0.50%	-	-		-	-		-	-
	Lu	FDA 71-64	0.50%	-	-		-	-		-	-
	T	FDA 71-64	0.50%	-	-		-	-		-	-

NOTE: TC = test compound
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2

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ACTIVATION PLATE TESTS

SPECIES: MONKEY

DATE: 8-30-74

Test	Organ	Compound	Concentration/plate	TA-1535 T-1	TA-1535 T-2	TA-1537 T-1	TA-1537 T-2	TA-1538 T-1	TA-1538 T-2
PC	Li	DMNA	25 µmoles	+	+				
		AAF	1.25 mg			+	+	+	+
	Lu	DMNA	25 µmoles	+/-	+/-				
		AAF	1.25 mg			-	-	-	-
	T	DMNA	25 µmoles	-	-				
		AAF	1.25 mg			-	-	-	-
SC	-	DMNA	25 µmoles	-	-				
	-	AAF	1.25 mg			-	-	-	-
	-	Saline	-	-	-				
	-	DMSO	<10%			-	-	-	-

NOTE:
PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2
DMSO = dimethyl sulfoxide
(c) = contamination present

Project No. 02468

Litton BIONETICS

ACTIVATION PLATE TESTS

SPECIES: MONKEY

DATE: 8-30-74

Test	Organ	Compound	Concentration	TA-1535		TA-1537		TA-1538	
				T-1	T-2	T-1	T-2	T-1	T-2
TC	Li	FDA 71-64	0.50%	-	-	-	-	-	-
	Lu	FDA 71-64	0.50%	-	-	-	-	-	-
	T	FDA 71-64	0.50%	-	-	-	-	-	-

NOTE:
TC = test compound
Li = liver
Lu = lung
T = testes
T-1 = trial 1
T-2 = trial 2

Project No. 02466

VIII. NON-ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS: POSITIVE AND SOLVENT CONTROL RESULTS

DATE: 8-16-74

Test	Indicator Strain	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	TA-1535	EMS	0.05 %	10.31	2,628	254.90
	TA-1537	QM	0.01 mg/ml	2.79	59	21.15
	TA-1538	NF	1.25 mg/ml	2.66	43	16.17
SC	TA-1535	SALINE	-	8.75	9	1.03
	TA-1537	SALINE	-	3.70	12	3.24
	TA-1538	DMSO	-	5.17	9	1.74

NOTE: PC = positive control
SC = solvent control
EMS = ethyl methanesulfonate
QM = quinacrine mustard
NF = nitrosofluorene
DMSO = dimthyl sulfoxide

NON-ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS

DATE: 8-16-74

Test	Indicator Strain	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
TC-H	TA-1535	FDA 71-64	0.50%	4.47 (51)	7	1.57
TC-L	TA-1535	FDA 71-64	0.50%	5.01 (57)	7	1.40
TC-H	TA-1537	FDA 71-64	0.50%	2.72 (74)	8	2.94
TC-L	TA-1537	FDA 71-64	0.50%	2.89 (78)	13	4.50
TC-H	TA-1538	FDA 71-64	0.50%	2.30 (44)	8	3.48
TC-L	TA-1538	FDA 71-64	0.50%	2.00 (39)	5	2.50 (c)

NOTE: TC-H = test compound high dose
TC-L = test compound low dose
(c) = contamination present
( ) = percent survival

Project No. 02468

IX. ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS: POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: MOUSE

DATE: 7-24-74

Strain TA-1535

Test	Organ	Compound	Concentration	Total Cells/ mlx10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	DMNA	100 µmoles/ml	5.71	2,233	391.07
	Lu	DMNA	100 µmoles/ml	4.99	49	9.82
	T	DMNA	100 µmoles/ml	7.25	5	0.69
SC	-	DMNA	100 µmoles/ml	10.55	11	1.04
	-	SALINE	-	5.80	11	1.90

DATE:

Strain TA-1537

Test	Organ	Compound	Concentration	Total Cells/ mlx10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	2.67	45	16.85
	Lu	AAF	1.25 mg/ml	1.85	11	5.95
	T	AAF	1.25 mg/ml	2.90	16	5.52
SC	-	AAF	1.25 mg/ml	2.11	6	2.84
	-	DMSO	-	1.83	11	6.01

DATE:

Strain TA-1538

Test	Organ	Compound	Concentration	Total Cells/ mlx10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	2.36	53	22.46
	Lu	AAF	1.25 mg/ml	2.40	16	6.67
	T	AAF	1.25 mg/ml	2.35	10	4.26
SC	-	AAF	1.25 mg/ml	2.75	13	4.73
	-	DMSO	-	2.26 (c)	6	2.65

NOTE: PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
DMSO = dimethyl sulfoxide

(c) = contamination present

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ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS

SPECIES: MOUSE

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
DATE: 7-24-74				Strain TA-1535		
TC	Li	FDA 71-64	H	6.35 (109)	16	2.52
		FDA 71-64	L	8.26 (142)	14	1.69
	Lu	FDA 71-64	H	5.93 (102)	5	0.84
		FDA 71-64	L	5.57 (96)	9	1.61
	T	FDA 71-64	H	5.29 (91)	11	2.03
		FDA 71-64	L	5.50 (95)	8	1.45
DATE: 7-25-74				Strain TA-1537		
TC	Li	FDA 71-64	H	2.73 (149)	54	19.78
		FDA 71-64	L	3.12 (170)	23	7.37
	Lu	FDA 71-64	H	1.16 (63)	8	6.90
		FDA 71-64	L	2.36 (129)	10	4.24
	T	FDA 71-64	H	3.02 (165)	17	5.63
		FDA 71-64	L	3.73 (204)	19	5.09
DATE: 7-26-74				Strain TA-1538		
TC	Li	FDA 71-64	H	2.41 (107)	9	3.73
		FDA 71-64	L	1.57 (69)	6	3.82
	Lu	FDA 71-64	H	2.40 (106)	8	3.33
		FDA 71-64	L	1.66 (73)	6	3.61
	T	FDA 71-64	H	4.12 (182)	16	3.88
		FDA 71-64	L	1.38 (61)	6	4.35

NOTES:
H = high dose
L = low dose
TC = test compound
Li = liver
Lu = lung
T = testes
(c) = contamination present
( ) = percent survival

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ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS: POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: RAT

DATE: 8-7-74

Strain TA-1535

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	DMNA	100 µmoles/ml	6.54	4,269	652.75
	Lu	DMNA	100 µmoles/ml	6.46	12	18.46
	T	DMNA	100 µmoles/ml	9.37	3	0.32
SC	-	DMNA	100 µmoles/ml	5.74	11	1.92
	-	SALINE	-	4.17	8	1.92

DATE: 8-8-74

Strain TA-1537

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	1.25	38	30.40
	Lu	AAF	1.25 mg/ml	6.50	33	5.08
	T	AAF	1.25 mg/ml	4.53	18	3.97
SC	-	AAF	1.25 mg/ml	5.62	6	1.07
	-	DMSO	-	5.88	9	1.53

DATE: 8-9-74

Strain TA-1538

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	2.84	165	58.01
	Lu	AAF	1.25 mg/ml	3.95	20	5.06
	T	AAF	1.25 mg/ml	5.09	26	5.11
SC	-	AAF	1.25 mg/ml	3.88	10	2.58
	-	DMSO	-	4.21	8	1.90

NOTE: PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
DMSO = dimethyl sulfoxide

(c) = contamination present

ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS

SPECIES: RAT

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
DATE: 8-7-71				Strain TA-1535		
TC	Li	FDA 71-64	H	3.21 (77)	8	2.49
		FDA 71-64	L	3.64 (87)	32	8.79
	Lu	FDA 71-64	H	4.35 (104)	8	1.84
		FDA 71-64	L	4.31 (103)	19	4.42
	T	FDA 71-64	H	4.08 (98)	20	4.90
		FDA 71-64	L	7.19 (172)	10	1.39
DATE: 8-8-74				Strain TA-1537		
TC	Li	FDA 71-64	H	0.55 (9)	25	45.45
		FDA 71-64	L	6.40 (109)	52	8.13
	Lu	FDA 71-64	H	4.57 (78)	12	2.63
		FDA 71-64	L	5.73 (97)	28	4.87
	T	FDA 71-64	H	7.56 (129)	10	1.32
		FDA 71-64	L	5.09 (87)	25	4.91
DATE: 8-9-74				Strain TA-1538		
TC	Li	FDA 71-64	H	2.00 (48)	24	12.00
		FDA 71-64	L	2.52 (60)	14	5.56
	Lu	FDA 71-64	H	2.96 (70)	14	4.73
		FDA 71-64	L	2.82 (67)	12	4.26
	T	FDA 71-64	H	1.68 (40)	17	10.12
		FDA 71-64	L	2.17 (51)	14	6.45

NOTES:
H = high dose
L = low dose
TC = test compound
Li = liver
Lu = lung
T = testes
(c) = contamination present
( ) = percent survival

Project No. 02468

Litton BIONETICS

ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS: POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: Rat

DATE: 9-4-74 (Repeated Dose) Strain TA-1535

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	DMNA	100 µmoles/ml	5.56	4,000	719.42
	Lu	DMNA	100 µmoles/ml			
	T	DMNA	100 µmoles/ml			
SC	-	DMNA	100 µmoles/ml			
	-	SALINE	-	4.01	11	2.74

DATE: 9-4-74 (Repeated Dose) Strain TA-1537

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	1.25	38	30.40
	Lu	AAF	1.25 mg/ml			
	T	AAF	1.25 mg/ml			
SC	-	AAF	1.25 mg/ml	1.91	17	8.90
	-	DMSO	-	1.75	13	7.43

DATE: Strain TA-1538

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml			
	Lu	AAF	1.25 mg/ml			
	T	AAF	1.25 mg/ml			
SC	-	AAF	1.25 mg/ml			
	-	DMSO	-			

NOTE: PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
DMSO = dimethyl sulfoxide

(c) = contamination present

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ACTIVATION SUSPENSION TESTS
WITH SALMONELLA INDICATOR STRAINS

SPECIES: Rat

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/ 10^8 Survivors
DATE: 9-4-74 (Repeated Dose)				Strain TA-1535		
TC	Li	FDA 71-64	H	3.99 (100)	29	7.27
			L			
	Lu		H			
			L			
	T		H			
			L			
DATE: 9-4-74 (Repeated Dose)				Strain TA-1537		
TC	Li	FDA 71-64	H	1.08 (62)	165	152.78
			L			
	Lu		H			
			L			
	T		H			
			L			
DATE:				Strain TA-1538		
TC	Li		H			
			L			
	Lu		H			
			L			
	T		H			
			L			

NOTES:
H = high dose
L = low dose
TC = test compound
Li = liver
Lu = lung
T = testes
(c)= contamination present
( ) = percent survival

Project No. 02468

ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS: POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: MONKEY

DATE: 9-5-74 — Strain TA-1535

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	DMNA	100 µmoles/ml	6.49	3,117	480.28
	Lu	DMNA	100 µmoles/ml	6.03	19	3.15
	T	DMNA	100 µmoles/ml	5.63	14	2.49
SC	-	DMNA	100 µmoles/ml	6.40	23	3.59
	-	SALINE	-	5.11	10	1.96

DATE: 9-17-74 — Strain TA-1537

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	5.47	84	15.36
	Lu	AAF	1.25 mg/ml	5.64	17	3.01
	T	AAF	1.25 mg/ml	4.57	19	4.16
SC	-	AAF	1.25 mg/ml	5.67	40	7.05
	-	DMSO	-	6.20	44	7.10

DATE: 9-27-74 — Strain TA-1538

Test	Organ	Compound	Concentration	Total Cells/ml x 10^8	his+ Revertants/ml	his+ Revertants/10^8 Survivors
PC	Li	AAF	1.25 mg/ml	6.68	305	45.66
	Lu	AAF	1.25 mg/ml	5.09	52	10.22
	T	AAF	1.25 mg/ml	5.15	33	6.41
SC	-	AAF	1.25 mg/ml	5.07	40	7.89
	-	DMSO	-	4.72	40	8.47

NOTE: PC = positive control
SC = solvent and chemical controls
AAF = 2-acetylaminofluorene
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes
DMSO = dimethyl sulfoxide

(c) = contamination present

Project No. 02468

ACTIVATION SUSPENSION TESTS WITH SALMONELLA INDICATOR STRAINS

SPECIES: MONKEY

Test	Organ	Compound	Concentration	Total Cells/ ml x 10^8	his+ Revertants/ ml	his+ Revertants/10^8 Survivors
DATE: 9-5-74				Strain TA-1535		
TC	Li	FDA 71-64	H	4.23 (83)	4	0.95
		FDA 71-64	L	5.81 (114)	23	3.96
	Lu	FDA 71-64	H	3.52 (69)	15	4.26
		FDA 71-64	L	5.86 (115)	26	4.44
	T	FDA 71-64	H	2.72 (53)	13	4.78
		FDA 71-64	L	5.88 (115)	26	4.42
DATE: 9-17-74				Strain TA-1537		
TC	Li	FDA 71-64	H	4.58 (73)	78	17.03
		FDA 71-64	L	5.92 (95)	46	7.77
	Lu	FDA 71-64	H	2.33 (38)	62	26.61
		FDA 71-64	L	5.92 (95)	24	4.05
	T	FDA 71-64	H	2.58 (42)	59	22.87
		FDA 71-64	L	6.46 (104)	25	3.87
DATE: 9-27-74				Strain TA-1538		
TC	Li	FDA 71-64	H	1.78 (38)	45	25.28
		FDA 71-64	L	4.28 (91)	52	12.15
	Lu	FDA 71-64	H	1.58 (33)	23	14.56
		FDA 71-64	L	4.87 (103)	51	10.47
	T	FDA 71-64	H	2.04 (43)	8	3.92
		FDA 71-64	L	3.25 (69)	46	14.15

NOTES: H = high dose
L = low dose
TC = test compound
Li = liver
Lu = lung
T = testes
(c) = contamination present
( ) = percent survival

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X. NON-ACTIVATION SUSPENSION TESTS WITH SACCHAROMYCES INDICATOR STRAIN D4

DATE: 7-29-74

			Strain D4				
Test	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
PC	EMS	1.0 %	4.71	479	315	101.70	66.88
SC	Saline	-	4.39	117	14	26.65	3.19

NOTE: PC = positive control
SC = solvent control
EMS = ethyl methanesulfonate
a = number x 10^5
b = number at 10^{-1} dilution

NON-ACTIVATION SUSPENSION TESTS WITH SACCHAROMYCES INDICATOR STRAIN D4

DATE: 7-29-74

			Strain D4				
Test	Compound	Concentration	Total Population Screened[a]	Number Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
TC	FDA 71-64	H	4.28 (97)	98	13	22.90	3.04
	FDA 71-64	L	4.30 (98)	221	73	51.40	16.98

NOTE: TC = test compound
H = high dose
L = low dose
a = number x 10^5
b = number at 10^{-1} dilution
( ) = percent survival

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XI. ACTIVATION SUSPENSION TESTS WITH SACCHAROMYCES INDICATOR STRAIN D4: POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: MOUSE DATE: 7-29-74

				Strain D4			
				Total Population Screened[a]	Number of Convertants[b]		Convertants Per 10^5 Survivors	
Test	Organ	Compound	Concentration		Ade^+	Try^+	Ade^+	Try^+
PC	Li	DMNA	150 μmoles/ml	2.90	237	67	81.72	23.10
	Lu	DMNA	150 μmoles/ml	3.23	68	27	21.05	8.36
	T	DMNA	150 μmoles/ml	3.38	61	11	18.05	3.25
SC	-	DMNA	150 μmoles/ml	2.89	42	13	14.53	4.50
	-	SALINE	-	2.75	89	6	32.36	2.18

NOTE: PC = positive control
SC = solvent and chemical controls
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes

a = number x 10^5
b = number at 10^{-1} dilution
(c) = contamination present

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ACTIVATION SUSPENSION TESTS WITH SACCHAROMYCES INDICATOR STRAIN D4

SPECIES: MOUSE DATE: 7-29-74

					Strain D4			
Test	Organ	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
TC	Li	FDA 71-64	H	3.33 (121)	139	48	41.74	14.41
		FDA 71-64	L	5.12 (186)	133	37	25.98	7.23
	Lu	FDA 71-64	H	3.17 (115)	119	48	37.54	15.14
		FDA 71-64	L	4.10 (149)	97	29	23.66	7.0
	T	FDA 71-64	H	2.93 (107)	103	26	35.15	8.87
		FDA 71-64	L	4.87 (177)	101	21	20.74	4.31

NOTE:
TC = test compound
H = high dose
L = low dose
Li = liver
Lu = lung
T = testes
a = number x 10^5
b = number at 10^{-1} dilution
(c) = contamination present
( ) = percent survival

ACTIVATION SUSPENSION TESTS
WITH SACCHAROMYCES INDICATOR STRAIN D4:
POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: RAT DATE: 8-5-74

					Strain D4			
Test	Organ	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
PC	Li	DMNA	150 µmoles/ml	2.44	124	74	50.32	23.77
	Lu	DMNA	150 µmoles/ml	2.73	73	10	26.74	3.59
	T	DMNA	150 µmoles/ml	2.72	85	9	31.40	3.42
SC	-	DMNA	150 µmoles/ml	3.29	74	13	22.52	3.98
	-	SALINE	-	2.66	99	10	37.22	3.76

NOTE: PC = positive control
SC = solvent and chemical controls
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes

a = number x 10^5
b = number at 10^{-1} dilution
(c) = contamination present

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ACTIVATION SUSPENSION TESTS WITH SACCHAROMYCES INDICATOR STRAIN D4

SPECIES: RAT DATE: 8-5-74

					Strain D4			
Test	Organ	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
TC	Li	FDA 71-64	H	2.98 (112)	125	20	41.95	6.71
		FDA 71-64	L	3.01 (113)	99	26	32.89	8.64
	Lu	FDA 71-64	H	2.87 (108)	112	18	39.02	6.27
		FDA 71-64	L	2.49 (94)	151	20	60.64	8.03
	T	FDA 71-64	H	3.06 (115)	96	12	31.37	3.92
		FDA 71-64	L	2.99 (112)	105	18	35.12	6.02

NOTE:
TC = test compound
H = high dose
L = low dose
Li = liver
Lu = lung
T = testes
a = number x 10^5
b = number at 10^{-1} dilution
( ) = percent survival

ACTIVATION SUSPENSION TESTS
WITH SACCHAROMYCES INDICATOR STRAIN D4:
POSITIVE AND SOLVENT CONTROL RESULTS

SPECIES: MONKEY DATE: 9-20-74

					Strain D4			
Test	Organ	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
PC	Li	DMNA	150 µmoles/ml	5.67	73	68	12.87	11.99
	Lu	DMNA	150 µmoles/ml	5.46	25	21	4.58	3.85
	T	DMNA	150 µmoles/ml	4.52	16	21(c)	3.54	4.65
SC	-	DMNA	150 µmoles/ml	5.45	25	21	4.59	3.85
	-	SALINE	-	5.46	9	15(c)	1.65	2.75

NOTE: PC = positive control
SC = solvent and chemical controls
DMNA = dimethylnitrosamine
Li = liver
Lu = lung
T = testes

a = number x 10^5
b = number at 10^{-1} dilution
(c) = contamination present

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ACTIVATION SUSPENSION TESTS
WITH SACCHAROMYCES INDICATOR STRAIN D4

SPECIES: MONKEY DATE: 9-20-74

				Strain D4				
Test	Organ	Compound	Concentration	Total Population Screened[a]	Number of Convertants[b] Ade+	Try+	Convertants Per 10^5 Survivors Ade+	Try+
TC	Li	FDA 71-64	H	4.84 (89)	54	86	11.16	17.77
		FDA 71-64	L	5.30(c) (97)	30	31	5.66	5.85
	Lu	FDA 71-64	H	5.60 (102)	42	59(c)	7.50	10.54
		FDA 71-64	L	5.16 (95)	37	0	7.17	-
	T	FDA 71-64	H	5.79 (106)	42	55	7.25	8.50
		FDA 71-64	L	5.86(c) (107)	37	0	5.31	-

NOTE:
TC = test compound
H = high dose
L = low dose
Li = liver
Lu = lung
T = testes
a = number x 10^5
b = number at 10^{-1} dilution
(c) = contamination present
( ) = percent survival

Project No. 02468

XII. SUMMARY OF RESULTS AND INTERPRETATION

Compound FDA 71-64, ferrous sulfate, was evaluated for genetic activity in a series of *in vitro* microbial assays. The results of this evaluation are as follows.

A. Salmonella Test Results

1. Plate tests

At a dose of 0.5% (w/v), ferrous sulfate did not exhibit any mutagenic activity in these assays.

2. Non-activation suspension tests

The results of these assays were negative.

3. Activation suspension tests

Results from these assays, especially with strain TA-1537 and to some extent strain TA-1538, showed increases in reversion frequencies in certain tests. The most consistant positive responses were obtained with strain TA-1537, a frameshift mutant. Fold increases in revertant over solvent control values at the high dose level with liver homogenates were 3.3, 29.7 and 3.1 for mouse, rat and monkey respectively. Strain TA-1538 showed 6.3- and 3.0-fold increases with rat and monkey liver homogenates respectively, at the high dose level (0.5%, w/v). Several other activation doses also exhibited increases with TA-1537 and TA-1538 with rat and monkey homogenates, although no consistant trends were evident. Strain TA-1535 was negative in all tests. It should be noted that the largest increases with both TA-1537 and TA-1538 were obtained with rat liver homogenates. In a repeat test with rat liver, essentially the same results were seen. TA-1535 showed little or no response whereas a 20.6-fold increase was seen with TA-1537. Several other repeat tests have been conducted with strain TA-1537 and all of these have been positive under activation conditions and negative under non-activation conditions.

B. Saccharomyces Test Results

1. Non-activation suspension tests

The low dose results show a substantial increase in gene conversion at the tryptophan locus with a smaller increase at the adenine locus. The high dose, however, showed no increase which suggests that the low dose results may be aberrant.

2. Activation suspension tests

The results of these tests also tend to support the other indications of genetic activity associated with ferrous sulfate. Increases in gene conversion at both loci were obtained in activation tests with mouse, rat and monkey tissues.

C. Conclusions

The results of this evaluation strongly suggest that ferrous sulfate possesses some genetic activity against microbial indicator organisms when tested in conjunction with metabolic activation systems. The facts that the two frame-shift mutants consistantly responded in several independent tests and that the rat tissue appeared consistantly most active provide the kinds of reproducibility that lend support to this conclusion. Less convincing are the positive results with strain D4 under conditions of activation. If the data is observed carefully, it can be seen that many of the increases with D4 occur in lung and testes activation tests. This is inconsistant with the Salmonella assays and is also inconsistant with the typical activation responses which usually show very little or no activation capacities for organs other than the liver. The D4 results should be viewed, therefore, with these facts in mind.

D. Additional Comments

It can be seen that the spontaneous reversion frequencies for the indicator strains are quite variable. This was expected and has been reported in the literature for both the Salmonella and yeast strains. Although cultures with quite low spontaneous frequencies can be selected, we feel that ranges of 1-5 x 10^{-8} for TA-1535 and 5-10 x 10^{8} for TA-1537 and TA-1538 are the mode.

The survival ranges, although not recorded, are also quite variable ranging from over 100% to less than 50% of the control values. It is our feeling that survival values are dependent on many factors and not only the toxicity of the test compound as determined in the initial survival curves.

Submitted by:

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APPENDIX

SUMMARY OF TESTS EVALUATING DMSO FOR GENETIC ACTIVITY IN *SALMONELLA* AND *SACCHAROMYCES*

Litton BIONETICS

COMPOUND DIMETHYSULFOXIDE

A. Suspension Tests

Test	Activation Species[a]	Activation Organ[b]	Salmonella Reversion Frequencies (x 10^{-8}) TA-1535	TA-1538	Saccharomyces D4 Conversion Frequencies (x 10^{-5}) Ade^+	Try^+
Non-activation						
Control (-C)	-	-	0.74	0.88	32.51	4.34
High Dose[c]	-	-	1.91	1.05	28.32	2.95
Low Dose[d]	-	-	0.53	1.37	40.73	0.49
Activation						
Control (+C)	-	-	1.80	0.36	38.27	2.47
Control (-C)	-	-	1.43	1.04	37.12	2.64
High Dose[c]	M	Li	0.34	1.07	47.77	2.75
	M	Lu	0.59	0.58	36.29	1.39
	M	T	0.62	0.30	34.35	1.94
Lose Dose[d]	M	Li	-	0.87	34.02	1.18
	M	Lu	0.43	3.14	42.30	1.40
	M	T	0.11	0.39	45.95	2.32

Note: (-C) = solvent control and (+C) = test chemical control without homogenate

a M = mouse, Mo = monkey, R = rat

b Li = liver, Lu = lung, T = testes

c Bacteria = 3%, Yeast = 5%

d Bacteria = 1.5%, Yeast = 2.5%

COMPOUND DIMETHYSULFOXIDE

B. Plate Tests

Test	Activation: Species[a]	Activation: Organ[b]	Salmonella Responses: TA-1535	TA-1537	TA-1538
Non-activation					
Control (-C)	-	-	-	-	-
Test compound (3%)	-	-	-	-	-
Activation					
Control (+C)	-	-	-	-	-
Control (-C)	-	-	-	-	-
Test compound (3%)	M	Li	-	-	-
	M	Lu	-	-	-
	M	T	-	-	-
	R	Li	-	-	-
	R	Lu	-	-	-
	R	T	-	-	-
	Mo	Li	-	-	-
	Mo	Lu	-	-	-
	Mo	T	-	-	-

Note: (-C) = solvent control and (+C) = chemical control without homogenate

a M = mouse
Mo = monkey
R = rat

b Li = liver
Lu = lung
T = testes